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Age-Associated Alteration of Blood Thiol-Group-Related Antioxidants in Smokers

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Total blood glutathione and nonglutathione free sulfhydryl compounds, glutathione peroxidase, and glutathione *S*-transferase in erythrocytes and plasma and also lipid peroxides in plasma were investigated in 48 male smokers and 42 male nonsmokers. We found that the level of total blood glutathione was significantly increased in young smokers (age < 40) but no such change was noted in aging smokers (age ≥ 40). The activities of glutathione peroxidase and glutathione *S*-transferase in plasma were significantly decreased in young smokers and the plasma levels of these two enzymes and nonglutathione free sulfhydryl compounds were more drastically decreased in aging smokers. The average concentration of plasma lipid peroxides of the aging smokers (2.76 ± 0.46 nmol/ml) was significantly higher than that of the aging nonsmokers (2.32 ± 0.41 nmol/ml, $P = 0.049$). On the other hand, the level of total blood glutathione was negatively correlated with the level of plasma lipid peroxides ($r = -0.305$, $P = 0.002$) and was positively correlated with the smoking index ($r = 0.307$, $P = 0.019$) of all the study subjects under age control. These results indicate that the activities of glutathione peroxidase and glutathione *S*-transferase declined to a great extent under smoking-mediated oxidative stress in the blood of both young and aging smokers. Moreover, the compensatory generation of total blood glutathione may effectively prevent plasma lipids from peroxidation in young smokers, although the activities of glutathione peroxidase and glutathione *S*-transferase in plasma were decreased. By contrast, total blood glutathione was inadequate for such protection in the aging smokers. We suggest that supplementation of thiol-group-related agents may be considered for the prevention or alleviation of oxidative stress in aging smokers, whose capability and capacity for the disposal of smoking-mediated free radicals and reactive oxygen species are compromised.

Key Words: **Thiol group-related antioxidants; lipid peroxidation; smoking; aging.**

INTRODUCTION

Nearly 50% of the deaths in the industrialized countries are a result of cardiovascular diseases, and cigarette smoking is thought to contribute to such mortality (Lakier, 1992). The effects of cigarette smoking on a variety of diseases, including cancer, emphysema, stroke, and cardiovascular disease, have been well documented (Aaron, 1983; Abbot *et al.*, 1986; Silverberg, 1984; Winniford, 1990). Tobacco smoke contains numerous compounds emitted as gases and condensed tar particles. Both the particulate and gas phases of cigarette smoke contain free radicals and reactive oxygen species (ROS) (Church and Pryor, 1985). Alteration in the levels of glutathione (GSH) and related antioxidants has been demonstrated to be highly associated with the smoking-mediated ROS and free radicals (Travis, 1987). GSH has been known to be widely distributed in human tissues and participates in an array of cellular defense mechanisms (Meister, 1989). It also protects cells from the toxic effects of xenobiotics and environmental pollutants by serving as a substrate in the removal of metabolic intermediates such as hydrogen peroxide, lipid peroxides, and organic hydroperoxides by GSH peroxidase (GSHPx). This thiol-group-mediated detoxification system can be activated during smoking and under altered physiological status of protein thiols, free thiols, and total GSH (Joshi *et al.*, 1988). GSH also forms conjugates with a variety of xenobiotics, particularly with the

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smoking-mediated organic free radials, by glutathione *S*-transferase (GST) and thereby effects the detoxification and excretion of ingested environmental pollutants and toxic or waste products.

Since smoking-elicited oxidative damage is systemic, we hypothesized that antioxidants in the blood plasma and erythrocytes (RBC) may be altered in smokers. The level of plasma lipid peroxides was monitored by the measurement of malondialdehyde (MDA), one of the end products of lipid peroxidation. The thiol-group-related antioxidants, including total blood GSH (tGSH) and non-GSH free sulfhydryl compounds (fSH), plasma GSHPx (pGSHPx), RBC GSHPx (rGSHPx), plasma GST (pGST), and RBC GST (rGST) were measured, respectively, in aging and young smokers as well as in nonsmokers.

MATERIALS AND METHODS

Subjects

Ninety healthy males of middle class without any systemic disease or long-term supplementation of vitamins were recruited in a special clinic of Kuang Tien General Hospital, Taichung, Taiwan. Forty-eight nonsmokers and 42 smokers were included in this study. Exsmokers or subjects with discontinuous smoking were excluded from either the smokers group or the nonsmokers group. Blood samples were taken with consent from each of the study subjects in the morning after overnight fasting before any medication or smoking. Ten milliliters of whole blood was withdrawn from an antecubital vein of each study subject and was delivered into a heparin-containing plastic tube. Plasma was immediately collected by centrifugation, divided into several aliquots, and stored at $-80°C$ in metal-free plastic tubes. The activities of antioxidant enzymes and the concentrations of small-molecular-weight antioxidants in the plasma were determined within 3 days. The cutoff point in age between young and aging smokers or nonsmokers was 40. The general data of the study subjects are summarized in Table 1.

TABLE 1
General Data of Young and Aging Study Subjects with and without Smoking

Subject parameters	Young nonsmokers[a] ($N = 19$)	Aging nonsmokers[a] ($N = 29$)	Young smokers[a] ($N = 16$)	Aging smokers[a] ($N = 26$)
Age (years)	34 ± 4	50 ± 8	32 ± 4	52 ± 11
BMI[b]	24 ± 2	25 ± 5	22 ± 3	24 ± 6
Fruits (U[c]/day)	2.6 ± 2.3	2.6 ± 2.3	2.1 ±2.2	2.0 ±2.3
Meat (U[c]/day)	4.3 ± 3.1	4.1 ± 3.4	4.8 ± 1.9	5.1 ±2.3
Leafy vegetables (U[c]/day)	1.6 ± 1.4	2.1 ± 1.1	1.6 ± 1.1	1.4 ±0.8
Smoking index[d]	0	0	214 ± 112	427 ±248

[a]Arithmetic mean ± SD.
[b]Body mass index.
[c]Unit defined by the Taiwan Provincial Sanitary Station of the Republic of China.
[d]Cigarette smoked per day × years of smoking. The average ages of starting smoking in young and aging groups were 17 ± 3 and 19 ± 4 years, respectively. The average numbers of cigarettes smoked per day in young and aging smokers were 15 ± 4 and 11 ± 6, respectively.

Determination of tGSH in Whole Blood

An aliquot of 0.05 ml of 10% perchloric acid (PCA) was added to 0.1 ml of whole blood to remove proteins by precipitation and centrifugation. Total free GSH in whole blood was measured with the recycling enzymatic assay, which employs glutathione reductase to induce a kinetic colorimetric reaction of DTNB (5,5′-dithiobis-(2-nitrobenzoic acid)) (Anderson and Meister, 1980). The rate of change of the absorbance at 412 nm was monitored at 30°C for 5 min. The concentration of tGSH in blood was calculated from a standard curve and is expressed as μg/ml.

Determination of Non-GSH fSH in Whole Blood

An aliquot of 0.1 ml of whole blood was deproteinated by the addition of 3.5% PCA followed by centrifugation. The supernatant was then added to 0.05 ml of 0.1 M DTNB solution. After thorough mixing, the mixture was left standing at room temperature. The final absorbance at 412 nm was recorded after 30 min reaction of DTNB with all the sulfhydryl compounds. The concentration of fSH was calculated by subtracting the concentration of GSH from that of tGSH. The blood concentration of fSH was expressed as μg/ml.

Measurement of GSHPx Activities in Plasma and Erythrocytes

A suitable portion of plasma or hemolyzed erythrocytes was added to 0.9 ml of 50 mM phosphate buffer (pH 7.0) containing 0.24 units of glutathione reductase and 1 mM GSH, and the mixture was incubated at 37°C for 10 min. NADPH was then added to a final concentration of 0.2 mM and the hydroperoxide-independent consumption of NADPH was monitored by the absorbance change at 340 nm for 3 min. Finally, the overall reaction was initiated by adding 100 μl of 12 mM *t*-butylhydroperoxide and

the decrease in the absorbance at 340 nm was then monitored for 5 min (Flohe and Gunzer, 1984). The enzyme activity of GSHPx was calculated from the hydroperoxide-dependent NADPH consumption rate and is expressed as U/liter or U/g Hb.

Measurement of GST in Plasma and Erythrocytes

The GST activity in the plasma or erythrocytes was measured using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate according to the method described by Habig *et al.* (1974). The assay mixture in 2 ml contained 0.2 M sodium phosphate buffer (pH 6.5), 0.95 mM CDNB, 0.95 mM GSH, and 0.5 ml plasma or lysed erythrocytes. The reaction was initiated by the addition of the electrophilic substrate CDNB and was monitored spectrophotometrically at 25°C by the increase of the absrobance at 340 nm. The background rate of absorbance change in the absence of the test sample was subtracted to correct for the nonenzymatic reaction. The GST activity is expressed as U/liter or U/g Hb.

Measurement of the Plasma Level of Lipid Peroxides

The plasma level of lipid peroxides was measured as malondialdehyde (MDA) using a method developed according to the principle described by Wong *et al.* (1987). Blood was withdrawn into a heparinized tube containing 10 μl of 2 mM butylated hydroxytoluene (BHT). The blood sample was immediately centrifuged to obtain plasma. A 20-μl aliquot of plasma was mixed with 2 ml of 0.1 N HCl, 0.3 ml of 10% phosphotungstic acid, and 1 ml of 0.7% 2-thiobarbituric acid (TBA). The mixture was heated for 60 min in boiling water and was then subjected to extraction of the MDA–TBA adduct with 5 ml 1-butanol. After centrifugation, the fluorescence intensity of the MDA–TBA complex in the 1-butanol layer was quickly measured in a fluorescence spectrophotometer using an excitation wavelength of 525 nm and an emission wavelength of 550 nm. The concentration of lipid peroxides in the plasma is expressed as MDA in nmol/ml. A calibration curve was constructed for each run by using 1,1,3,3-tetraethoxypropane as the standard.

Statistical Analysis

The Mann–Whitney *U* test was applied in the statistical analyses of data. A partial correlation analysis was also applied in the search for causal relationships between parameters under age control. All of the statistical procedures were performed using the SPSS statistical package.

RESULTS

There were no differences in the age, body mass index (BMI), and dietary intake of fruits and vegetables between smokers and nonsmokers (Table 1). As shown in Table 2, the average concentration of tGSH in the blood of the young smokers (238 ± 19 μg/ml) was significantly higher than that of nonsmokers (213 ± 33 μg/ml, $P = 0.023$). However, the blood level of tGSH was not significantly increased in aging smokers. In young smokers, the activities of pGSHPx (647 ± 56 U/liter vs nonsmokers 729 ± 118 U/liter, $P = 0.041$) and pGST (6.74 ± 0.86 U/liter vs nonsmokers 7.77 ± 1.81 U/liter, $P = 0.039$) were found to be significantly decreased. In addition, the levels of pGSHPx (545 ± 107 U/liter vs nonsmokers 650 ± 141 U/liter, $P = 0.037$), pGST (6.12 ± 1.45 U/liter vs nonsmokers 7.36 ± 0.85 U/liter, $P = 0.031$), rGST (4.71 ± 1.09 U/g Hb vs nonsmokers 5.90 ± 1.49 U/g Hb, $P = 0.029$), and fSH (109 ± 24 μg/ml vs nonsmokers 137 ± 27 μg/ml, $P = 0.017$) were found to decrease more profoundly in the aging smokers. The average concentration of plasma MDA in the aging smokers (2.76 ± 0.46 nmol/ml) was higher than that

TABLE 2

The Concentrations of Antioxidants in the Plasma and RBC of Smokers and Nonsmokers in Young and Aging Groups

	Nonsmoker[a]	Smoker[a]	*P*value[b]
Young group (age ≤ 40)	$N = 19$	$N = 16$	
pGSHPx (U/liter)	729 ± 118	647 ± 56↓	0.041[c]
rGSHPx (U/g Hb)	57 ± 13	63 ± 12	0.537
pGST (U/liter)	7.77 ± 1.81	6.74 ± 0.86↓	0.039[c]
rGST (U/g Hb)	5.34 ± 1.36	6.05 ± 2.67	0.748
tGSH (μg/ml)	213 ± 33	238 ± 19↑	0.023[c]
fSH (μg/ml)	140 ± 34	144 ± 17	0.086
MDA (nmol/ml)	2.20 ± 0.49	2.42 ± 0.37	0.162
Aging group (age > 40)	$N = 29$	$N = 26$	
pGSHPx (U/liter)	650 ± 141	545 ± 107↓	0.037[c]
rGSHPx (U/g Hb)	66 ± 14	60 ± 18	0.912
pGST (U/liter)	7.36 ± 0.85	6.12 ± 1.45↓	0.031[c]
rGST (U/g Hb)	5.90 ± 1.49	4.71 ± 1.09↓	0.029[c]
tGSH (μg/ml)	193 ± 35	212 ± 45	0.151
fSH (μg/ml)	137 ± 27	109 ± 24↓	0.017[c]
MDA (nmol/ml)	2.32 ± 0.41	2.76 ± 0.46↑	0.049[c]

Note. ↑Significantly increased. ↓Significantly decreased.
[a]Arithmetic mean ± SD.
[b]Comparison by Mann-Whitney *U* test.
[c]$P < 0.05$.

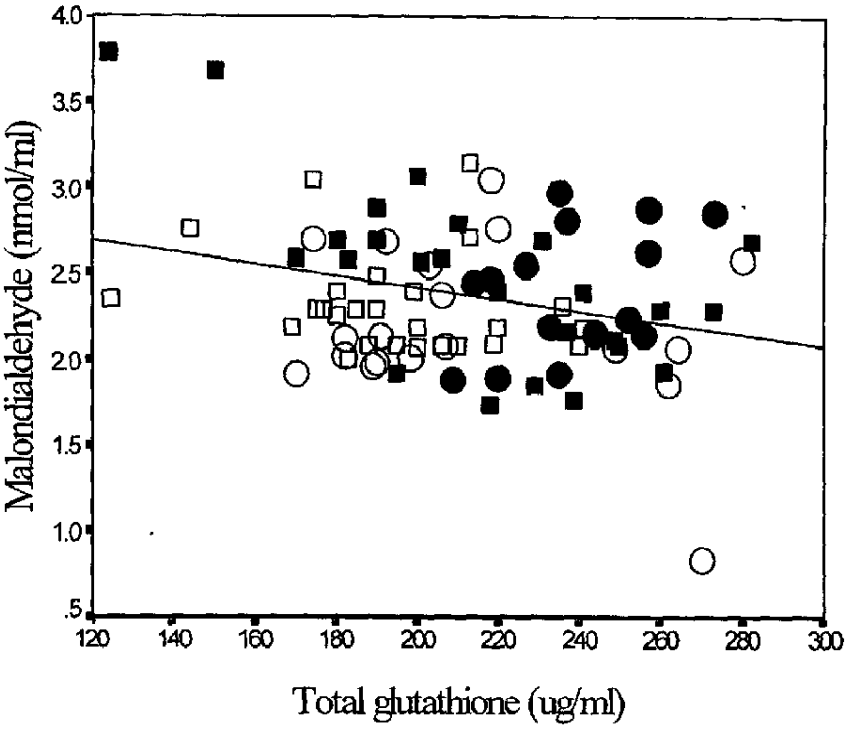

FIG. 1. Data dispersion and the regression line between the blood level of total glutathione and that of plasma MDA in both smokers and nonsmokers. There are 90 data sets, including 48 male smokers and 42 male nonsmokers in the scattered plots. (○) Young nonsmokers, (●) young smokers, (□) aging nonsmokers, (■) aging smokers. The P value calculated by partial correlation analysis of the data under age control is 0.002. The correlation coefficient was calculated to be -0.305.

in the aging nonsmokers (2.32 ± 0.41 nmol/ml, $P = 0.049$). However, there was no difference in plasma MDA between young smokers and young non-smokers. Figure 1 shows that the blood level of tGSH was negatively correlated with the level of plasma MDA ($r = -0.305$, $P = 0.002$) of all the study subjects under age control. Moreover, analysis of the correlations between the levels of thiol-group-related antioxidants and MDA revealed that the group of aging smokers displayed a significant negative correlation between blood tGSH and plasma MDA concentrations ($r = -0.626$, $P = 0.017$). In addition, we found that there is a significant positive correlation between pGSHPx and MDA in young smokers ($r = 0.571$, $P = 0.026$) (Table 3). Figure 2 shows that there was a positive correlation between blood level of tGSH and smoking index ($r = 0.307$, $P = 0.019$).

TABLE 3

Correlations between the Levels of Plasma Thiol-Group-Related Antioxidants and Malondialdehyde in Each Group under Age Control[a]

	Young nonsmokers[b]	Aging nonsmokers[b]	Young smokers[b]	Aging smokers[b]
pGSHPx vs. MDA	−0.194/0.473	0.108/0.690	0.571/0.026[c]	−0.555/0.851
pGST vs. MDA	0.007/0.979	−0.223/0.400	−0.277/0.318	−0.022/0.940
tGSH vs. MDA	−0.414/0.110	−0.181/0.503	0.417/0.121	−0.626/0.017[c]

[a]Comparison by the analysis of partial correlation.
[b]Correlation coefficient/P value.
[c]$P < 0.05$.

DISCUSSION

Many smoking-mediated prooxidants can trigger free radicals and ROS-mediated oxidative damage to human tissues, including lipid peroxidation in the cell membrane. The thiol-group-related antioxidants, including fSH, tGSH, GSHPx, and GST, constitute one of the major free radical scavenging systems involved in the disposal of the smoking-mediated prooxidants (Eiserich *et al.*, 1995). GSH is a physiological antioxidant that is widely distributed in human tissues and participates in various antioxidant mechanisms, particularly in the detoxification of xenobiotics (Meister, 1989). It protects cells from the toxic effects of ROS and other organic hydroperoxides by serving as a substrate for GSHPx and GST during the removal of metabolic intermediates and waste products of xenobiotics, drugs, chemicals, and environmental pollutants. Cantin *et al.* (1987) reported that cigarette smokers have a significantly higher level of GSH in the epithelium lining fluid of the lower tracheal tract, whose alveolar surface suffers from enhanced oxidative stress elicited by smoking. Toth and co-workers (1986) found that the RBC of cigarette smokers have a higher content of GSH to protect the endothelial cells from damage by ROS than the RBC of the nonsmokers. Michelet *et al.* (1995) disclosed that the blood level of tGSH in smokers is positively correlated with the number of cigarettes smoked per day. In an *in vitro* study, Eiserich *et al.* (1995) found that exposure of a GSH solution (0.1 mmol/liter) to three puffs of cigarette smoke resulted in complete GSH depletion and that cigarette smoke promoted the consumption of protein thiols and enhanced lipid peroxidation in blood plasma. These observations imply that the thiol-group-related antioxidants quickly respond to the smoking-related prooxidants and are poorly regenerated *in vitro*. In this study, we found that increased blood levels of tGSH and fSH could effectively prevent the enhancement of plasma lipid peroxidation in young smokers but not in aging smokers (Tables 2 and 3, Fig. 1). Moreover, the increased blood level of tGSH was positively correlated with the smoking index in the young, but not in aging, smokers (Fig. 2). It is noteworthy that the blood level of tGSH was negatively correlated with the concentration of plasma lipid peroxides

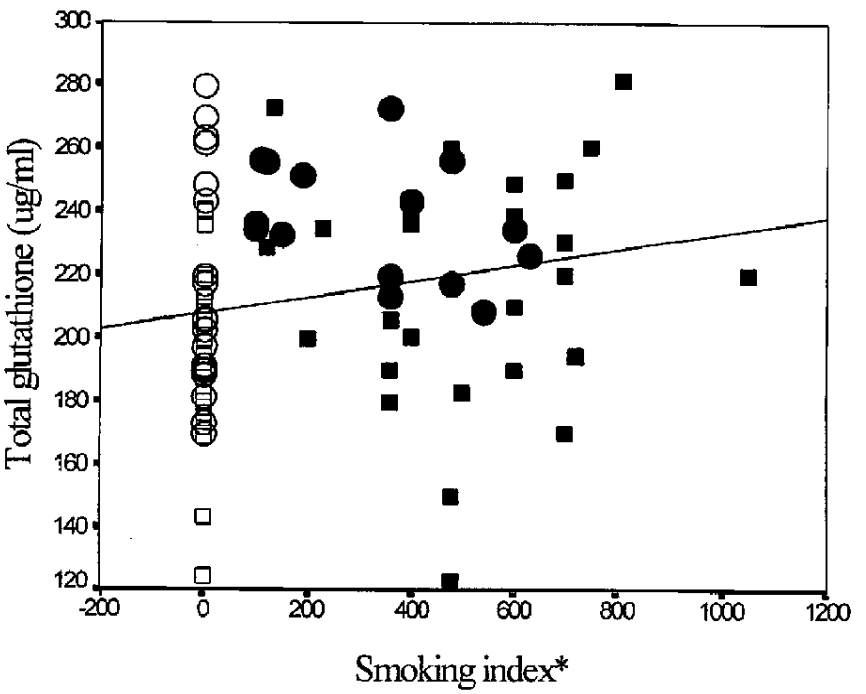

FIG. 2. Data dispersion and the regression line between smoking index and blood level of total glutathione. There are 90 data sets, including 48 male smokers and 42 male nonsmokers in the scattered plots. (*) Cigarette smoked per day × years of smoking. (○) young non-smokers, (●) young smokers, (□) aging nonsmokers, (■) aging smokers. The P value is 0.019, as calculated by partial correlation analysis of the data under age control. The correlation coefficient is 0.307.

(Table 3). Furthermore, we found that the blood concentration of tGSH exhibited a 9–11% decrease in both smoking and nonsmoking aging subjects (mean age was 51). In an earlier study, Al-Turk *et al.* (1987) observed a 56% decrease of GSH content in the RBC of elderly subjects (mean age was 70). These results suggest that the thiol-group-related compounds in the blood circulation are maintained at sufficiently high levels by the compensatory induction of biosynthesis of GSH from the liver and peripheral tissues in young smokers but not in aging smokers. Therefore, age is an important factor in determining the strength of antioxidant defense against the smoking-mediated prooxidants. This is due, at least partly, to the aging-associated decline or impairment of the regeneration of thiol-group-containing antioxidants.

Since many smoking-mediated mutagens or carcinogens bear electrophilic and/or nucleophilic structural moieties, the activity of GST that catalyzes the conjugation of xenobiotics with GSH is important in reducing the susceptibility of tissues to the damage caused by these deleterious compounds (Habig *et al.*, 1974). In smokers, the level of polycyclic aromatic hydrocarbon–DNA adduct, an evidence of the genotoxicity of cigarette smoke, was found to correlate negatively with the level of gene expression of GST (Bell *et al.*, 1993; Grinberg-Funes *et al.*, 1994). Moreover, the incidences of smoking-associated cancers were found to be higher in subjects with a mutated GST gene (Alexandrie *et al.*, 1994). In the present study, we found that both pGST and rGST are significantly decreased in the blood of aging smokers. This finding is consistent with the notion that GST plays a very important role in the detoxification of smoking-mediated xenobiotics and organic free radicals.

GSHPx can reduce organic peroxides and protect cell membranes against lipid peroxidation (Meister, 1989). Cigarette smoke has been demonstrated to inhibit GSHPx activity in the rat lung (Joshi *et al.*, 1988). Abou-Seif (1996) also observed that the activity of GSHPx is significantly decreased in the blood of smokers. Hulea et al. (1995) further disclosed that the activity of pGSHPx is decreased in aging subjects (mean age was 65) and is depleted to a greater extent in smokers. In this study, we found that the plasma level of GSHPx was decreased in the young smokers and declined even more dramatically in aging smokers. Interestingly, we found that there exists a positive correlation between plasma GSHPx and MDA in the group of young smokers, but not in the group of aging smokers (Table 3). These findings suggest that the enzyme activity of GSHPx changed in parallel with the plasma MDA level under the oxidative stress elicited by cigarette smoke in young smokers.

In conclusion, we found that both pGSHPx and pGST declined in the first place under smoking-elicited oxidative stress in both young and aging smokers. This implies that these two enzymes in the blood act as the early responders to cigarette smoke so as to scavenge smoke-mediated ROS and organic free radicals. Furthermore, we demonstrated that the compensatory generation of tGSH can effectively prevent plasma lipids from peroxidation, which may be effected by the coordinated action of pGST and pGSHPx, in young smokers. However, this phenomenon was not observed in aging smokers. These results suggest that supplementation of thiol-group-related agents (e.g., *N*-acetylcysteine) may be considered for the prevention or alleviation of oxidative stress imposed on aging smokers, whose capabilities and capacities in the scavenging of smoking-mediated free radicals and ROS are compromised in tissue cells and blood circulation.

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